# Red Blood Cell Immune Complex Binding Capacity in Children with Sickle Cell Trait (HbAS) Living in *P. falciparum* Malaria Holoendemic Region of Western Kenya

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*Authors' contributions*

This work was carried out in collaboration between all authors. Author WO helped in study design, conducted all experiments, helped in data analysis and drafted the manuscript, author JAS designed the study, directed the work, drafted the manuscript, helped in data analysis and data interpretation, author BBAE helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, author JRA helped in study design, data analysis, data interpretation  and helped in drafting of the manuscript, author MMO helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. All authors read and approved the final manuscript.

**Research Article**

## ABSTRACT

**Aims:** Malaria infection leads to the formation of circulating immune complexes (CICs) which have been implicated in the pathogenesis of complicated malaria which includes severe malarial anemia. Children with sickle cell trait (HbAS) are less predisposed to getting severe manifestations of malaria. We carried out a study to determine the competence of the red blood cells (RBCs) of children with HbAS to bind immune complexes (ICs) and compared this with normal hemoglobin (HbAA).
**Methods:** Children (aged 0-192 months) were enrolled in a nested case controlled study

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# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*

| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| **08 DEC 2012** | | **00-00-2012 to 00-00-2012** |

| 4. TITLE AND SUBTITLE | 5a. CONTRACT NUMBER |
|---|---|
| **Red Blood Cell Immune Complex Binding Capacity in Children with Sickle Cell Trait (HbAS) Living in P. falciparum Malaria Holoendemic Region of Western Kenya** | 5b. GRANT NUMBER |
| | 5c. PROGRAM ELEMENT NUMBER |
| 6. AUTHOR(S) | 5d. PROJECT NUMBER |
| | 5e. TASK NUMBER |
| | 5f. WORK UNIT NUMBER |

| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) | 8. PERFORMING ORGANIZATION REPORT NUMBER |
|---|---|
| **US Army Medical Research Unit,,Kenya Medical Research Institute, Kisumu, Kenya, , ,** | |

| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | 10. SPONSOR/MONITOR'S ACRONYM(S) |
|---|---|
| | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

**12. DISTRIBUTION/AVAILABILITY STATEMENT**
**Approved for public release; distribution unlimited**

**14. ABSTRACT**
**Malaria infection leads to the formation of circulating immune complexes (CICs) which have been implicated in the pathogenesis of complicated malaria which includes severe malarial anemia. Children with sickle cell trait (HbAS) are less predisposed to getting severe manifestations of malaria. We carried out a study to determine the competence of the red blood cells (RBCs) of children with HbAS to bind immune complexes (ICs) and compared this with normal hemoglobin (HbAA).**

**15. SUBJECT TERMS**

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT **unclassified** | b. ABSTRACT **unclassified** | c. THIS PAGE **unclassified** | **Same as Report (SAR)** | **12** | |

**Standard Form 298 (Rev. 8-98)**
Prescribed by ANSI Std Z39-18

conducted in Kombewa Division, Kisumu West District, Kenya. Based on hemoglobin (Hb) type, children were stratified into those with HbAS (n=47) and HbAA (n=69). The 47 HbAS individuals were matched to 69 HbAA of similar age. The children were further categorized into three cohorts (0-12, 13-48 and 49-192 months). Immune complex binding capacity (ICBC) was quantified using a FACScan flow cytometer under normal and reduced oxygen saturation.

**Results:** The mean immune complex binding capacity for the HbAS cells was significantly higher than that of HbAA cells (*P=0.0191*) under normal oxygen saturation or under reduced oxygen saturation (*P*=0.0050). When a matching variable (UNIANOVA) was done to control for age, gender, the presence or absence of malaria parasitaemia, the binding capacity was again significantly higher for the HbAS than for HbAA under normal oxygen saturation (*P*=0.025) and under reduced oxygen saturation (*P*=0.003).

The binding capacity was lowest in the 7-12 months age group for both HbAS and HbAA; however, the overall picture showed that HbAS individuals had higher immune complex binding capacity than HbAA in all the age cohorts.

**Conclusion:** These results demonstrate that the protection afforded by HbAS against severe manifestations of malaria may be partly due to higher immune complex binding capacity of the HbAS compared to the HbAA cells. This high binding capacity may lead to the mopping up of ICs formed during malaria attacks and therefore protect these cells from deposition and subsequent destruction.

## 1. BACKGROUND

Severe anemia is one of the most serious complications of *Plasmodium falciparum* malaria that occurs predominantly in children in the first 3 years of life and is an important cause of childhood morbidity and mortality in sub-Saharan Africa [1, 2]. The pathogenesis of anemia during malarial infections is not fully understood. Some of the mechanisms that have been implicated include suppression of erythropoeisis [3], increased destruction of RBCs as a result of parasite replication [4] and immune-mediated accelerated destruction of parasitized as well as non-parasitized erythrocytes [5] . Despite all this work, the pathogenesis of severe malarial anemia is not well understood; recent studies have shown that ICs are deposited on RBCs in children with severe malarial anemia [6-9]. These ICs have been shown to play an important role in the pathogenesis of severe malarial anemia. Heterozygous sickle cell trait individuals (HbAS) are relatively protected from severe manifestations of malaria such as anemia [10] the underlying mechanisms of this protection are however not well understood but are thought to be due to several mechanisms [11]. Given the important role played by immune complex deposition in the pathogenesis of severe malarial anemia, this study investigated whether there are differences in immune complex binding capacity between erythrocytes from HbAS and HbAA individuals that could partly explain the protection from severe anemia in individuals with HbAS.

*P. falciparum* infection leads to severe immunostimulation with generation of several soluble plasmodial antigens [12]. The formation of CICs is the physiological consequence of the binding of antibodies that are produced in response to the different antigens and under normal circumstances; mechanisms for their efficient and rapid clearance exist in mammals. In some abnormal circumstances, their accumulation in tissue or circulation induces pathological consequences. This is seen in disorders such as rheumatoid arthritis or

systemic lupus erythematosus [13-15]. Under normal circumstances ICs activate complement either through the classical or the alternative pathways. In humans, the subsequent covalent binding of C3b or C4b to the complexes allows them to bind to complement receptor 1 (CR1, CD35) expressed on erythrocytes. Once bound to erythrocytes via CR1, the C3b-containing ICs are carried to the liver and spleen, where they are removed by macrophages in a process known as the transfer reaction and the erythrocytes return to circulation [16]. Thus, low CR1 expression levels on erythrocytes may result in poor immune complex clearance capacity, which then predisposes one to immune complex-mediated complications. The ICs that are not bound to erythrocytes can deposit in tissues and on endothelium where they continue to activate complement and stimulate leukocyte production of nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-$\alpha$) by leukocytes; in turn contributing to development of severe anemia. Children with severe malaria-associated anemia and cerebral malaria have significant higher CICs than their respective controls and this predisposes their RBCs to increased erythrophagocytosis due to a relative deficiency of CR1 and CD55. This deficiency is thought to result in impaired clearance of ICs from the circulation [6, 9].

## 1.1 Immune Complex Binding Capacity of E-Complement Receptor-1

As indicated above, Erythrocyte CR1 binds ICs and acts as an inert shuttle transporting them to the liver and the spleen where they are removed by macrophages. This multi-valent binding is favored by the known clustering of CR1 and by the multiple binding sites on each CR1. Almost 50% of CR1 are distributed in clusters of greater than or equal to three units compared to less than 15% on the surface of polymorphonuclear leukocytes. Although the numbers of CR1 clusters vary from cell to cell, the mean number of clusters correlates significantly with the mean number of CR1 per cell. This cluster could explain the high binding efficiency of C3b coated IC to erythrocytes. The clustering of E CR1 makes CR1 a privileged site on erythrocytes and prevents phagocytosis of erythrocytes opsonized via CR1 [17]. The number of CR1 molecules is known to decrease with aging of the erythrocytes in normal individuals and also in certain disease states such as systemic lupus erythematosus, HIV and some hemolytic anemia. In these conditions, the erythrocyte clearance mechanism for the ICs is interfered with and the ICs deposit on tissues such as the skin, lungs and kidney resulting in disease [13]. Recent studies in our laboratory have associated increased levels of CICs with severe malaria anemia [6, 7] Patients with severe malaria anemia have been shown to have low CR1 [7, 9] and this may result in poor immune complex clearance capacity through phagocytosis in the spleen and liver. Owuor *[18]* have further demonstrated that deficiencies in red cell CR1 in children with severe malaria anemia were accompanied by a marked decline in immune complex binding capacity. Collectively, these data indicate that immune complex binding capacity by erythrocytes influences the clearance capacity in *P. falciparum* malaria patients and may determine an individuals' susceptibility to severe malarial anemia. The immune complex binding capacity by erythrocytes of sickle cell trait individuals has not been examined for association with resistance to severe malaria anemia.

## 2. MATERIALS AND METHODS

### 2.1 Study Site and Design

The study site was Kombewa Division, in Kisumu West District, Nyanza Province, in western Kenya. Kombewa is situated about 35 kilometres west of Kisumu town and has previously

been used as a site for many epidemiological studies in both adults and children [19, 20]. Kombewa borders Lake Victoria and has a population of about 65,000 people. Malaria transmission in this area occurs all year round with peak seasons following the long rains (March to May) and the short rains (October to December). The annual inoculation rates are estimated to be 31.1 infective bites per person per year [20]. This study was carried out between the months of October and December 2004.

## 2.2 Study Population

Forty seven (47) HbAS individuals aged 0-192 months were matched to 69 individuals with HbAA of similar age (± 2 months or ± 24 months for those below or more than 96 months, respectively) at a ratio of 1:1 or 1:2. Since many acute or chronic conditions including malaria, HIV infection and others are known to interfere with the parameters under investigation such as complement regulatory proteins [7, 9, 21, 22], the exclusion criteria included evidence of malnutrition, immuno compromised status, severe anemia (Haemoglobin 5.0 g/dL); bacterial infection such as pneumonia; malignancy; and blood transfusion within 3 months preceding the study. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well.

## 2.3 Ethical Consideration

Recruitment of study participants and procedures were in accordance with all applicable regulations. Informed consent was obtained from all participants or parents/guardians of children. This study was reviewed and approved by the Kenya National Ethical Review Committee of the Kenya Medical Research Institute and by the Human Subjects Research Review Board of the Office of the Surgeon General, U.S. Army.

## 2.4 Preparation of Immune Complexes

50 µL of 49 mg/ml rabbit anti-BSA (Sigma-Adrich, St.Louis, MO) and 3 µL of 5 mg/ml BSA-FITC (Accurate Chemical and Scientific Corp., Westbury, NY) were added to 950 µL of RPMI1640 (Sigma-Aldrich). This combination was noted to be the point of equivalence in preliminary experiments. The mixture was incubated at 37ºC for 1 hr and overnight at 4ºC. The next day, the IC preparation of soluble and insoluble IC was aliquoted and stored at -20ºC.

## 2.5 Measurement of Immune Complex Binding Capacity of Erythrocytes

For immune complex opsonization, 5µL of stock IC or RPMI 1640 (unstained control) was incubated in a total volume of 100 µl containing 30% AB+ serum in wells of a 96-well plate. For a negative control, a separate set of wells contained IC plus 10mM EDTA. Following incubation at 37ºC for 30 minutes with constant rocking motion, 100µL 1% hematocrit suspension of freshly thawed red cells from each study participants or from a standard aparasitemic control in RPMI 1640 was added to each of the wells of the above 96-well plate. This was followed by further incubation for 30 minutes at 37ºC. The erythrocytes were then washed twice in 200µL of ice cold RPMI, re-suspended in PBS containing 1% paraformaldehyde, and stored at 4ºC until acquisition. After gating, the erythrocyte fluorescein isothiocyanate (FITC) fluorescence was measured using logarithmic amplification and the positive cutoff was set using unstained cells. The percent of the positive cells (IC

Binding) was calculated based on this cutoff. To control for day to day variation, the IC binding capacity was normalized to the mean IC binding capacity of the red cell standard used throughout using the mean of the Mean Fluorescence Intensity (MFI) of the red cell standard using the formula:

$$CorrMFIs = MFIs \times MFIcmean/MFIc,$$

Where "CorrMFIs and MFIs are the corrected and uncorrected sample MFI respectively, 'MFIcmean" is the mean of all the MFI values of the standard control and "MFIc" is the MFI of the control obtained in parallel with the sample.

## 2.6 Statistical Analysis

Statistical analyses were performed using SPSS for windows version 15.0 software (SPSS Inc, Chicago, IL, USA) and the graphs were done using Graph Pad Prism 5. The mean IC binding capacity per red cell are presented graphically for each age group as box plots, where the box represents boundaries between the 25th and 75th percentile, the line through the box represents median and whiskers the 10th and 90th percentile limits. Analysis of variance (ANOVA) was used to detect differences across age groups adjusting for factors and covariates. The independent samples t-test was used for comparisons of normal continuous data between two groups, while the Chi-square ($\chi^2$) and Mann-Whitney U tests were utilized to examine differences between proportions and for pair wise comparisons of medians, respectively. Bivariate logistic regression analysis was carried out to determine the Odds Ratio (OR) and the 95% confidence interval (CI) for mean IC binding capacity per red cell between HbAS and HbAA .The General Linear Method (GLM) was used to test between subject effects. The Chi-square ($\chi^2$) test was used to compare proportions across groups. All tests were two-sided with     0.05.

## 2.7 Deoxygenation of the RBCs for Assay

An equal amount of RBCs in wash buffer was added to freshly prepared disodium hydrogen phosphate ($Na_2HPO_4$, FW 142g) 0.114M and sodium dithionite ($Na_2S_2O_4$ FW 174.1g) 0.114M at a ratio of 2:3, filter sterilized through a 0.22um filter [23]. The disodium hydrogen phosphate was prepared from a stock solution while the sodium dithionite was prepared fresh every day. The RBCs were incubated at 37ºC for 1 hour and then washed twice with wash buffer before running the assays side by side. This duration of treatment with the dithionite was found to give the maximal sickling for HbAS RBCs. This procedure was done to see the effect of reduced oxygen saturation on the parameters under investigation.

## 3. RESULTS

### 3.1 Differences in IC Binding Capacity between HbAS and HbAA Red Cells under Normal and Reduced Oxygen Saturation

We measured the mean IC binding capacity for these cells under both normal and reduced oxygenation saturation. The mean IC binding capacity under normal and reduced oxygen saturation is shown (Fig. 1). The data are presented as box-and-whisker plots. For each group, the horizontal line in the middle of the box marks the median of the sample. The box represents the interquartile range and the central 50% of the data falls within the range of the box. The whiskers are the vertical lines extending up and down from each box and they represent the upper and the lower 25% of the data. The mean immune complex binding capacity for the HbAS cells (52.5[SD=6.4]) was higher than HbAA cells (48.1[SD=11.6]).

This difference was statistically significant with $P = 0.0191$. Deoxygenation of the RBCs did not appear to have an effect on the mean immune complex binding capacity of the RBCs. Compared to the HbAA, The mean immune complex binding capacity under deoxygenated for the HbAS cells (52.6=7[SD=7.8]) was still higher than HbAA cells (48.1[SD=11.6]). This difference was statistically significant with P =0.005 (Fig. 1).
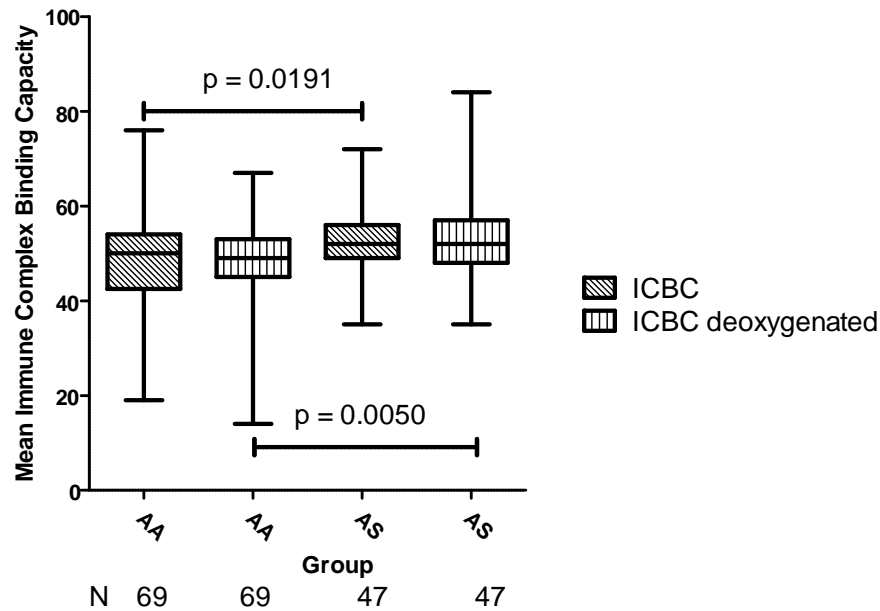


**Fig. 1. Immune complex binding capacity by haemoglobin type**

We used the univariate analysis of variance with matching variable using the general linear model procedure to compare the immune complex binding capacity between HbAS and HbAA. The immune complex binding capacity was again significantly higher for the HbAS than HbAA ($P = 0.025$). This was also true under reduced oxygen saturation ($P = 0.003$).

### 3.2 Differences in Mean IC Binding Capacity in HbAS and HbAA Cells in Each Age Cohort

In order to see whether the red cells IC binding capacity varied with age, we measured the IC binding capacity in the various age groups. The mean IC binding capacity per erythrocyte under normal conditions (Fig. 2) was observed to be generally higher in HbAS than HbAA individuals in all the age cohorts. Despite the general trend of high immune complex binding capacity in the HbAS compared to HbAA across the age groups, the difference in the cohorts 0-12, 13-48 and 49-192 months were statistically not different ($P = 0.151$, 0.280 and 0.315 respectively). There was a weak positive correlation between the IC binding capacity and the age cohort r (116) = 0.198, $P = 0.034$.

### 3.3 Differences in Mean IC Binding Capacity in HbAS and HbAA Red Cells in Each Age Cohort under Reduced Oxygen Saturation

The mean IC binding capacity under reduced oxygen saturation was higher in the HbAS compared to HbAA across the various age cohorts but the difference between cohorts 0 –

12, 13 – 48 and 49 – 192 months were statistically not significant (*P* values 0.084, 0.264 and 0.068 respectively) (Fig. 3).
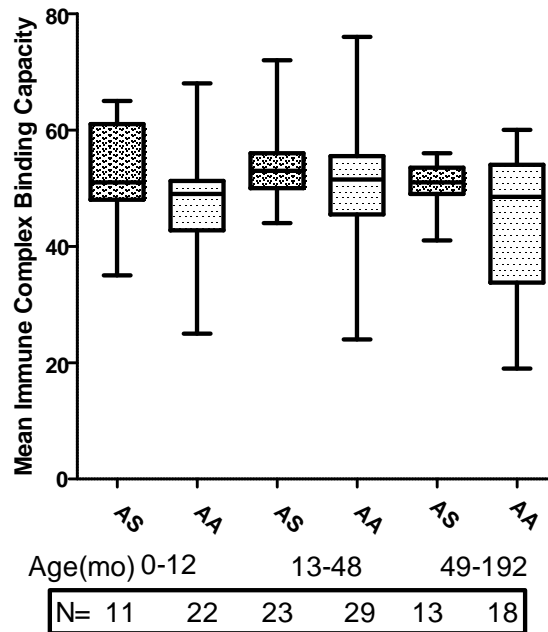


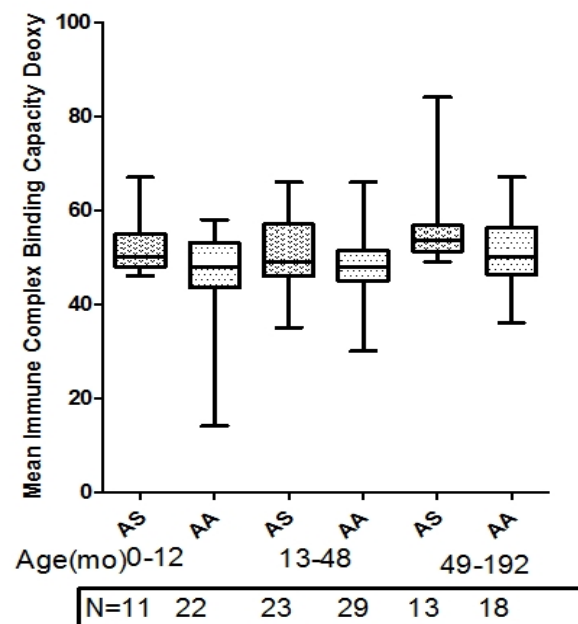**Fig. 2. Mean IC binding capacity in HbAS and HbAA cells by age cohort**



**Fig. 3. Mean IC binding capacity in HbAS and HbAA cells in each age cohort under reduced oxygen saturation**

## 4. DISCUSSION

In malaria, a number of ICs are generated [6, 24] . These CICs may lead to end organ damage by depositing on erythrocytes and other organs like the kidney [25-27] and have been associated with severity of malaria especially in children [18, 28]. Studies from our laboratory have shown that ICs are elevated in severe malarial anemia at the time of diagnosis. These ICs stimulate pro-inflammatory cytokines that play a role in the pathogenesis of severe malaria.

The ability to bind CICs is critical for mopping up the circulating complexes. High ability to mop up ICs leads to less chances of getting severe manifestations of malaria. The immune complex binding capacity for HbAS cells were much higher than for the HbAA cells ($P$=0.0191). This was also true after deoxygenation ($P$=0.0050). It is apparent that the HbAS cells bind more ICs than the HbAA cells and this may lead to increased mopping up of the ICs generated during malaria infections and therefore prevent severe manifestations of malaria. After deoxygenation, the apparent increase in the immune complex binding capacity of the HbAS erythrocytes could be due to increased exposure of the binding sites. This may be due to conformational changes in the CR1 molecules resulting in the exposure of more binding sites.

The geographical distribution of blood group O has been shown to be consistent with a selection pressure by *P. falciparum* in favor of blood group O similar to what is seen in individuals with HbAS [29, 30]. Erythrocyte CR1 has also been proposed to be an important determinant of malaria susceptibility in several investigations [7, 9, 31] and RBCs of children with severe malaria associated  anemia have acquired deficiencies of the complementary regulatory proteins CR1 and CD55 which affects their RBCs to bind CICs which may predispose these RBCs to complement mediated damage [18]. The  AB antigen and CR1 share the same binding site in parasite and this may also explain the reduced susceptibility of HbAS to severe manifestations of malaria [30, 32].

Like in other studies, age correlated with immune complex binding capacity with high levels in the $\leq$12 months age cohort. Thereafter, the binding capacity decreased with the advancing age. Similar findings have been reported [8, 33].  It was also noted that, the lowest level of immune complex binding capacity corresponded to the lowest levels of CR1 in the population [8] as well as the age where the majority of children get severe malarial anemia [7, 8, 33, 34].

## 5. CONCLUSIONS

We report for the first time that the mean immune complex binding capacity for the HbAS cells is higher than HbAA cells both under normal and reduced oxygen saturations. This is also true when a matching variable is factored in. Like in other studies, the mean immune complex binding capacity was lowest in the >6-12 months age group for both HbAS and HbAA in our study. However, the overall picture showed that HbAS individuals had higher immune complex binding capacity than HbAA in all the age cohorts. These results taken together may translate into protection of the HbAS individuals against severe manifestations of malaria.

## DISCLAIMER

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of Defense.

## ACKNOWLEDGMENTS

## COMPETING INTERESTS

There is no conflict of interest for any of the authors of the manuscript due to commercial or other affiliations. The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute and the institutional review board at the Walter Reed Army Institute of Research.

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